



# Non-replicating Newcastle Disease Virus as an adjuvant for DNA vaccine enhances antitumor efficacy through the induction of TRAIL and granzyme B expression

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## ABSTRACT

The potential of non-replicating Newcastle Disease Virus (NDV) as an adjuvant for DNA vaccination remains to be elucidated. To assess the therapeutic effects of DNA vaccine (HPV-16 E7 gene) adjuvanted with NDV, female C57/BL6 mice were inoculated with murine TC-1 cells of human papillomavirus (HPV)-related carcinoma, expressing human papillomavirus 16 (HPV-16) E6/E7 antigens, and immunized with DNA vaccine alone or pre-treated with NDV. One week after third immunization, Cytotoxic T lymphocytes (CTLs), splenocyte proliferation, cytokine balance (IFN- $\gamma$ , IL-4 and IL-12 secretions) and intratumoral expression of cytotoxicity related proteins in tumor lysates were investigated.

The results showed that treatment with non-replicating NDV prior to DNA vaccine induced tumor-specific cytolytic and splenocyte proliferation responses. The levels of cytokines IL-12, IL-4 and IFN- $\gamma$  after treating with combined E7-DNA -non-replicating NDV (NDV-DNA Vaccine) were significantly higher than those of control groups. The intratumoral granzyme B and Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL)-mediated apoptosis was also significantly increased. Tumor therapeutic experiments showed that the NDV pretreatment could reduce the tumor progression of established E7-expressing TC-tumors. Taken together these data suggest that the significant antitumor responses evidenced during treatment with non-replicating NDV prior to DNA vaccine are due, in part, to strong E7-induced cellular immunity and enhanced expression of cytotoxicity related proteins in the tumor microenvironment. These observations indicated the potential of non-replicating NDV as an adjuvant for enhancing therapeutic DNA vaccines -induced immunity and antitumor responses.

**Abbreviations:** APC, antigen-presenting cell; CTL, cytolytic T lymphocyte; DC, dendritic cells; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HPV, human papilloma virus; ICD, immunogenic cell death; IFN- $\gamma$ , interferon  $\gamma$ ; IL-4, interleukin 4; IL-12, interleukin 12; LDH, lactate dehydrogenase; LPA, lymphocyte proliferation assay; mDC, myeloid dendritic cells; MTT, 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, thiazolyl-blue; NDV, Newcastle Disease Virus; NLR, nucleotide-binding oligomerization domain NOD-like receptor; OD, optical density; pDC, plasmacytoid dendritic cell; PHA, phytohemagglutinin; PBS, phosphate-buffered saline; PRR, pattern recognition receptors; PAMP, pathogen-associated molecular patterns; RLR, retinoic acid-inducible gene I (RIG-I)-like receptors; RPMI, 1640 Roswell Park Memorial Institute (name of the medium); SPF, specific pathogen-free; TAA, tumor associated antigens; Th, T helper; TLR, toll-like receptors; Trail, tumor necrosis factor related apoptosis inducing ligand

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## 1. Introduction

Cervical cancer is the second most common cancer in women worldwide and the leading cause of cancer deaths (Waggoner, 2003). Chronic infection of the reproductive tract with human papillomavirus (HPV) is usually the cause of cervical cancer, and the continued expression of the E6 and E7 oncogenes of high-risk HPVs is required for the maintenance of the proliferative state of cervical cancer cells (Bosch and de Sanjose, 2003).

Immunotherapy has become an increasingly appealing therapeutic strategy for patients with cancer. Immunotherapies against tumors include various approaches, ranging from cancer vaccines to oncolytic viruses that have been evaluated in both pre-clinical and clinical investigations (Farkona et al., 2016).

Among different types of cancer vaccines, DNA vaccines have emerged as an attractive strategy for antigen-specific cancer immunotherapy due to their simplicity, stability, safety, and their capacity to induce a broad spectrum of cellular and humoral immune responses (Yang et al., 2014).

Despite all the benefits, DNA vaccines suffer from limited therapeutic effects due to low transfection efficiency in vivo. Therefore, it is important to devise new methods to augment the intensity and duration of vaccine-induced immune responses and to confer a protective anti-tumor immune response. Development of novel immunostimulants is likely to meet many challenges in modern vaccinology (Gableh et al., 2016; Sajadian et al., 2014).

HPV therapeutics like DNA vaccines are well tolerated to induce antigen-specific immunity through the viral antigen processing and presentation in antigen presenting cells (Lin et al., 2010). Although immunostimulatory agents are a promising approach to improve cancer treatment, the role of non-human viruses in anti-tumor immunomodulation has been investigated, and potent immunomodulatory activities have been reported for some viruses (Prestwich et al., 2008).

Treatment of a tumor with a virus leads to immunogenic cancer cell death (ICD) induction, resulting in the release of danger signals or pathogen/damage-associated molecular patterns (P/DAMPs) (Workenhe and Mossman, 2014), along with the reduced intratumoral immunosuppressive environment and increased cross-priming of tumor-associated antigens (Alemany, 2014). The treatment can convert an immunosuppressive tumor microenvironment to an immunostimulatory status. Considering these immunostimulatory actions, it has been hypothesized that whether non-replicating virus can be combined with other immunotherapeutic strategies including cancer vaccines (Bastin et al., 2016).

Newcastle Disease Virus (NDV) is a RNA virus with long known cancer specific inducing apoptotic cell death activities (Szeberenyi et al., 2003). Post-oncolytic activity of NDV, i.e. ICD, suggests this virus as a potent agent to overcome tumor-induced immunosuppressive environment (Schirmacher, 2015). As the viruses may have side effects to normal cells, tumor selective oncolysis of the viruses is not always possible (Wong et al., 2010). Although the antitumoral effects of live or inactivated NDV have been studied, we describe here the adjuvant effects of non-replicating NDV in combination with cancer vaccine against TC-1 tumor induction. The NDV components like cytosolic viral long RNA, mRNA, fusion protein (F) and hemagglutinin neuraminidase (HN) are all involved in and can trigger tumor cell death through either immune induction or immune independent apoptosis (Mansour et al., 2011; Ni et al., 2010). The NDV-induced ICD optimizes presentation of tumor associated antigens (TAA) and facilitates antitumor immune responses (Schirmacher, 2015).

Naturally occurring NDV strains induced limited tumor-specific immunity, but play an important role in modulation of the host immune response and recognition of vaccine antigens (Zamarin et al., 2009). It has been shown that immunized mice with NDV LaSota strain leads to production of anti-viral neutralizing anti-bodies, which cause superior

anti-tumoral effects and prolonged median survival of treated animals (Chaurasiya et al., 2018; Ricca et al., 2018). It is also known that heterologous prime-boosting strategy, in which the prime response is stimulated by a virus enhances antiviral immune responses (Bridle et al., 2009). Therefore, in the current study we have aimed to evaluate cancer immunotherapy using a therapeutic tumor-associated antigen expressing E7 DNA vaccine after priming with lentogenic LaSota (La) NDV strain (Romer-Oberdorfer et al., 2003) as adjuvant. The results of the current study showed that HPV-16 E7 DNA vaccine-induced tumor protection was significantly enhanced by pre-treating the tumor with a non-replicating NDV strain.

## 2. Materials and methods

### 2.1. NDV preparation

The LaSota strain of NDV was obtained from Razi Institute of Serum and Vaccine Research Center, Tehran, Iran. Virus was propagated of 10 day-old embryonated specific pathogen-free (SPF) eggs and purified from the chorioallantoic fluid. The number of infectious virus particles in a preparation was evaluated by using plaque forming assay at 40–72 h post infection (Kournikakis and Fildes, 1988). NDV strain LaSota ( $10^8$  pfu) was inactivated at UV light power of 50 mW/cm<sup>2</sup> for 5 min at 5 cm distance from the UV lamp just before use. In addition, the completeness of UV inactivation of NDV strain LaSota was confirmed by plaque forming assay on Vero cell line (Ghrichi et al., 2013; Walter et al., 2012). The results showed that The UV-inactivated NDV did not form any plaques on Vero cells.

### 2.2. Cell lines

The murine lung cancer cell line, TC-1, was derived from primary lung epithelial cells from C57BL/6 mice. The cell line was immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene and purchased from the National Cell Bank of Iran (Pasteur Institute of Iran).

The cell line was maintained in complete RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD), 2.5 mM 2-mecaptoethanol (Invitrogen, Carlsbad, CA), 0.5 mM sodium pyruvate (Sigma Aldrich, Germany), 2 mM L-glutamate (Invitrogen, Carlsbad, CA), and 100 U/mL of penicillin- 100 mg/L streptomycin (Sigma, St. Louis, MO, USA) (Moeini et al., 2017).

The murine thymoma cell line EL4 (murine T-cell lymphoma of haplotype H-2b) was cultured (at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air) in RPMI 1640 supplemented with 10% FBS.

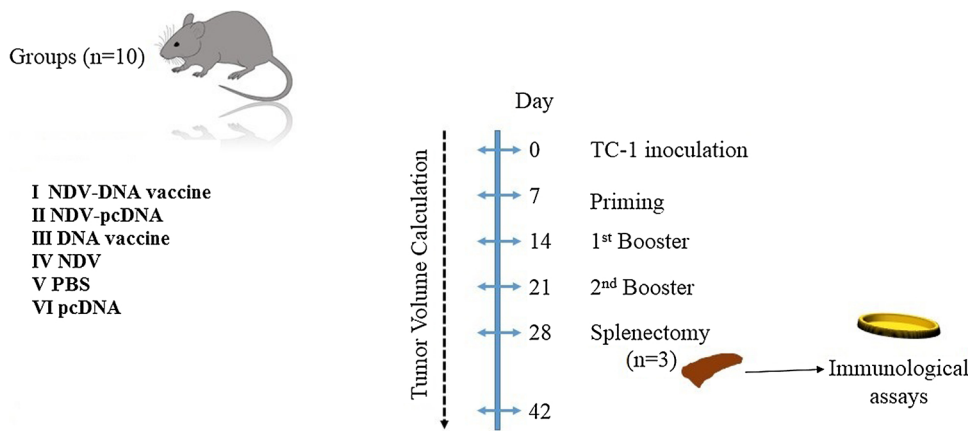
### 2.3. Animal bearing tumor

Six - to eight-week-old Female C57BL/6 (H2b) mice were obtained from the Institute Pasture of Iran (Tehran, Iran). Mice were housed for 1 week before the experiment, given free access to food and water, and maintained in a 12:12-h light: dark cycle. All experiments were carried out in accordance with the Animal Care and Use Protocol of Golestan University of Medical Sciences of Iran.

For tumor induction (Sajadian et al., 2014), six groups of C57BL/6 mice (n = 10) were inoculated by subcutaneous (s.c.) injection in the right flank with  $3 \times 10^5$  TC-1 cells in 100  $\mu$ l PBS.

### 2.4. DNA vaccine and NDV administration

Plasmids containing HPV-16 E7 gene were constructed for mammalian expression in our laboratory previously. Plasmid constructs were verified by DNA sequencing and gene expression (Sajadian et al., 2014). Stocks of endotoxin free DNA vaccine and vector control plasmids (pcDNA3.1) in 0.1 M PBS were purified for vaccination using the



**Fig. 1.** Schematic overview of study design: The time scale at the top represents the days after TC-1 inoculation, vaccination, immunological assays and tumor volume calculation. At time zero mice were inoculated with TC-1 tumor. Mice were immunized by subcutaneous injection with different treatments. Three mice in each of 6 groups were sacrificed at the 28-day time points. Spleen were collected for immunological assays.

EndoFree® Plasmid Maxi Kit (Qiagen, Hilden, Germany) and dissolved in endotoxin free Tris-EDTA (TE Buffer - 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) (Sigma, St. Louis, MO, USA).

One week after tumor induction, animals were randomly divided into six groups (n = 10 in each group). The first two groups were injected with NDV subcutaneously at a dose of  $10^5$  pfu/mouse. Twelve hours later, the E7-DNA vaccine (NDV-DNA vaccine, group I) or pcDNA<sub>3</sub> control vector (NDV-pcDNA, group II) were inoculated (E7 DNA vaccine, 100 µg in 100 µl using subcutaneous administration). The same procedure of immunization employing the same dose of treatments was used for repeat immunizations on days 14 and 21. Each animal in groups III (DNA vaccine) and IV (NDV) were respectively administered with 100 µg DNA-E7 vaccine and  $10^5$  pfu NDV with the same protocol. PBS and pcDNA<sub>3</sub> were injected according the same protocol into V and VI group as control groups (PBS and pcDNA groups) (Fig. 1).

Tumor growth was monitored by visual inspection and palpation one week after TC-1 inoculation. The average mean of all tumor volumes were calculated and reported in cubic millimeters. Tumor diameters were measured in a blinded, coded fashion twice a week using two orthogonal dimensions with electronic digital calipers. The tumor volume was calculated as: (longest diameter) × (shortest diameter)  $\times$  0.5. The formula given for the tumor volume is according to Carlsson's formula (Carlsson et al., 1983). Mice were euthanized once tumors exceeded a diameter of 1 cm or became necrotic to the animals. Tumor growth was followed for a period of 6 weeks after the inoculation. All values were expressed as means  $\pm$  S.D. Three mice per group were sacrificed one week after the last boost and the spleens were removed aseptically for immunological tests. Schematic overview of study design has been shown in Fig. 1.

## 2.5. Cytokine assay

One week after the third immunization, spleens from the immunized mice were removed and red blood cells were cleared by incubation in lysis buffer (0.15 M NH<sub>4</sub>Cl, 1 mM KHCO<sub>3</sub>, 0.1 mM Na<sub>2</sub>EDTA, pH 7.2) for 5 min at 37 °C. Mononuclear cells at a concentration of  $2 \times 10^5$  cells per well were incubated in 24-well plates for 3 days in RPMI1640 supplemented with 10% FBS, 1% L-glutamine, 1% HEPES, 2.5 mM 2-mercaptoethanol, and pulsed with E7-specific H-2Db CTL epitope at a concentration of 1 µg/ml (Biomatik, Ontario, Canada, > 99% purity) (Gableh et al., 2016) and at 37 °C in 5% CO<sub>2</sub>. IFN- $\gamma$ , IL-4 and IL-12 secretion were evaluated 48 h after stimulation. Culture supernatant samples were collected and assayed for the presence and titer of cytokine-specific polyclonal antibodies conjugated to horseradish peroxidase were detected using TMB (tetramethylbenzidine) substrate using commercially available Quantikine Mouse IFN- $\gamma$ , IL-4 and IL-12 assays (R&D Systems Inc., Minneapolis, Minn, USA) according to the manufacturer's instruction. The plates

were read at 450 nm, and values were expressed as optical densities. All tests were performed in triplicate.

## 2.6. Lymphocyte proliferation assay (LPA)

One week after third immunization, a single-cell suspension of mononuclear cells obtained from immunized mice was used for the lymphocyte proliferation assay (LPA). The preparations were cultured in complete medium, and incubated in the presence of E7-specific H-2Db CTL epitope (1 µg/ml) per well at 37 °C in 5% CO<sub>2</sub>. Five µg/ml T cell mitogen phytohemagglutinin (PHA) (Sigma Chemicals, St. Louis, MO, USA) was used as the positive control. After 3 days, 5 µg/ml of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemicals, St. Louis, MO, USA) was added to each well and incubated for 5 h at 37 °C in 5% CO<sub>2</sub>. DMSO (100 µl) was added to dissolve formed formazan crystals. Then, optical density was read at 540 nm (and reference at 630 nm) and the results expressed as a stimulation index (SI). The SI was determined as follows:

$$SI = \frac{OD_{stimulated\ culture}}{OD_{unstimulated\ culture}}$$

All tests were done in triplicate for each mouse.

## 2.7. Cytotoxicity assay

One week after last booster, the mice (three mice of each group) were sacrificed and their splenocytes were isolated. For each sample obtained from individual mice, single-cell suspensions of mononuclear cells (used as the effector cells) were cultured in RPMI 1640 medium with washed EL4 target cells (a mouse lymphoma cell line derived from C57BL/6 (MHC- H-2b); ATCC TIB-39, from the National Cell Bank of Iran (Pasteur Institute, Tehran)) at various effector-to-target cell (E/T) ratios (25:1, 50:1, 100:1) and in 96-well flat-bottom plates for 4 h in complete medium. For preparation of the target cells, EL4 cells were stimulated with H2b-restricted epitope E7-specific CTL epitope 49RAHNIVTF57 at a concentration of 1 µg/ml (Biomatik, Ontario, Canada, > 99% purity) (specific antigen) and then incubated for 4 h. The ectodomain of matrix protein 2 (M2e) of influenza A virus synthetic peptide was used as irrelevant peptide and a negative control. After centrifugation, the supernatants (50 µl/well) were transferred to the 96-well flat-bottom plates (Corning, Corning, NY, USA), and lysis of target cells was determined by measuring lactate dehydrogenase (LDH) release using a LDH cytotoxicity detection kit (Takara Company, Shiga, Japan). Several controls were used for the cytotoxicity assay. The 'high control' was used for measuring total LDH release from the target cells (all EL4 cells were lysed with medium containing 1% Triton X-100). The 'low control' was used for measuring natural release of LDH from the target cells (which was obtained by adding EL4 cells only to the medium).

The LDH-mediated conversion of tetrazolium salt into a red formazan product was measured at 490 nm (Gableh et al., 2016; Naderi et al., 2013). The percentage of specific cytotoxicity was determined by the following formula:

$$\text{Cytotoxicity \%} = \left[ \frac{(\text{Test sample} - \text{effector}) - \frac{\text{low control}}{\text{high control} - \text{low control}}}{\text{high control} - \text{low control}} \right] \times 100$$

## 2.8. Preparation of tumor homogenate

To evaluate the cytokine levels in tumor microenvironment, the tumor homogenate was prepared for all experimental tumor-bearing mice. Briefly, TC-1 tumors were harvested and tumor tissue extracts were prepared by mechanical homogenization and sonication of 100 mg of tumor in 500 mL of lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, and 1 mM phenyl-methane-sulfonyl fluoride [PMSF] (Sigma-Aldrich, St. Louis, Missouri, USA). The samples were then homogenized and centrifuged and supernatants were removed and filtered through a 0.45 µm filter; afterwards, the protein concentration was measured by the Bradford method (Sajadian et al., 2014).

## 2.9. Intratumoral cytokine assay

To detect the cytotoxic expression in the tumor microenvironment, tumor homogenates were used for the ELISA assay. For the assay, protein concentration of tumor homogenate samples were adjusted to 2 mg/ml with PBS and then the level of intratumoral mouse TRAIL (Tumor Necrosis Factor Related Apoptosis Inducing Ligand) was determined using an mouse DuoSet ELISA Kit (R&D Systems Inc., Minneapolis, MN, USA). Granzyme B expression was also evaluated using a Mouse Granzyme B ELISA Ready-SET-Go (eBioscience, Inc. San Diego, CA, USA) according to the manufacturer's instruction.

## 2.10. Statistical analysis

To compare results between the different groups one-way ANOVA was used. The statistical software SPSS version 16.0.0 was used for data analyses. Differences were considered statistically significant at  $p < 0.05$ .

## 3. Results

### 3.1. Lymphocyte proliferation

Evaluation of lymphocyte proliferation in the experimental groups was carried out with a MTT/ELISA-based method. Two DNA vaccinated groups (DNA vaccine and NDV-DNA vaccine groups) showed significant differences when compared with NDV, NDV-pcDNA ( $P < 0.05$ ), PBS and pcDNA control groups ( $P < 0.001$ ). Immunization with combined E7-DNA -non-replicating NDV (NDV-DNA vaccine) significantly increased lymphocyte proliferation compared with NDV and DNA treated groups ( $P < 0.001$ ). The results also revealed that DNA vaccine alone significantly increased stimulation index compared to the NDV and NDV-pcDNA groups ( $P < 0.05$ ). There was no significant difference between the control groups (NDV-pcDNA, NDV, PBS and pcDNA) ( $P > 0.05$ ) (Fig. 2). These results suggest that inoculated with non-replicating NDV can enhance the antigen-specific T-cell response of DNA vaccine.

### 3.2. CTL assay

T lymphocyte cytolytic activity was measured with release of Lactate dehydrogenase assay (LDH). The effector: target cell ratio up to

the maximum ratio of 100:1 was used in the present study (Fig. 3A). The results of LDH release assay as a criterion of CTL activity index indicate that injection of combined E7-DNA -non-replicating NDV (NDV-DNA vaccine group) significantly augmented CTL activity compared with the control groups (NDV-pcDNA, NDV, PBS and pcDNA) ( $p < 0.001$ ). Also treatment with combined E7-DNA-non-replicating NDV (NDV-DNA vaccine group) led to a 22% increase of CTL responses versus DNA vaccine alone ( $P < 0.01$ ). No significant difference between the control groups (NDV, PBS and pcDNA) was observed (Fig. 3B). Irrelevant peptide as a negative control showed no influence on E7-specific cytotoxicity between different groups. The results showed the NDV ability to enhance the antitumor cytolytic activity of DNA vaccine.

### 3.3. Cytokine assay

To further determine the effect of NDV on DNA vaccine-induced immune response, the expression of cytokines were measured in spleen cells. Assessment of the cytokines in the experimental groups indicate that combined E7-DNA -non-replicating NDV (NDV-DNA vaccine group) or immunization with DNA vaccine alone significantly increased IFN- $\gamma$  and IL-12 secretion compared with the control groups (NDV-pcDNA, NDV, PBS and pcDNA3) ( $p < 0.001$ ) (Fig. 4 A, C). In spleen cells, higher IL-4 production was seen in DNA vaccinated mice compared with negative control groups (PBS and pcDNA) (Fig. 4B), whereas this was not seen compared with NDV and NDV-pcDNA treated mice. Furthermore, stimulated IFN- $\gamma$ , IL-12 and IL-4 production were higher in the NDV-DNA vaccine group compared to DNA vaccine group ( $P \leq 0.001$ ). Therefore, immunization with DNA vaccine combined with NDV as adjuvant promoted IFN- $\gamma$ , IL-4 and IL-12 production, resulting in an enhanced cellular immune response.

### 3.4. Tumor therapy experiment

In the therapeutic model, all mice that had formed a palpable tumor (at day 7) were vaccinated on days 7, 14 and 21 with the indicated regimens and followed for tumor growth and survival over six weeks.

Over a total of six weeks, it was demonstrated that all mice in the NDV-DNA vaccine group controlled tumor growth significantly better than all other groups (Fig. 5). Compared with a moderate inhibitory effect of DNA vaccine on tumor growth, NDV adjuvant increased the antitumor effect of the DNA vaccine at 42 days post-tumor- inoculation ( $p < 0.001$ ). Note that the mean size of the tumors ( $\sim 185 \text{ mm}^3$ ) is lower in NDV-DNA vaccine group than in DNA-vaccinated mice ( $\sim 365 \text{ mm}^3$ ). In contrast, the use of NDV as a monotherapy and NDV -pcDNA administration had no inhibitory activity. These data indicate that vaccination with DNA vaccine in combination with low-dose non-replicating Newcastle disease virus induced a strong immune response, which translated into a protective antitumor response.

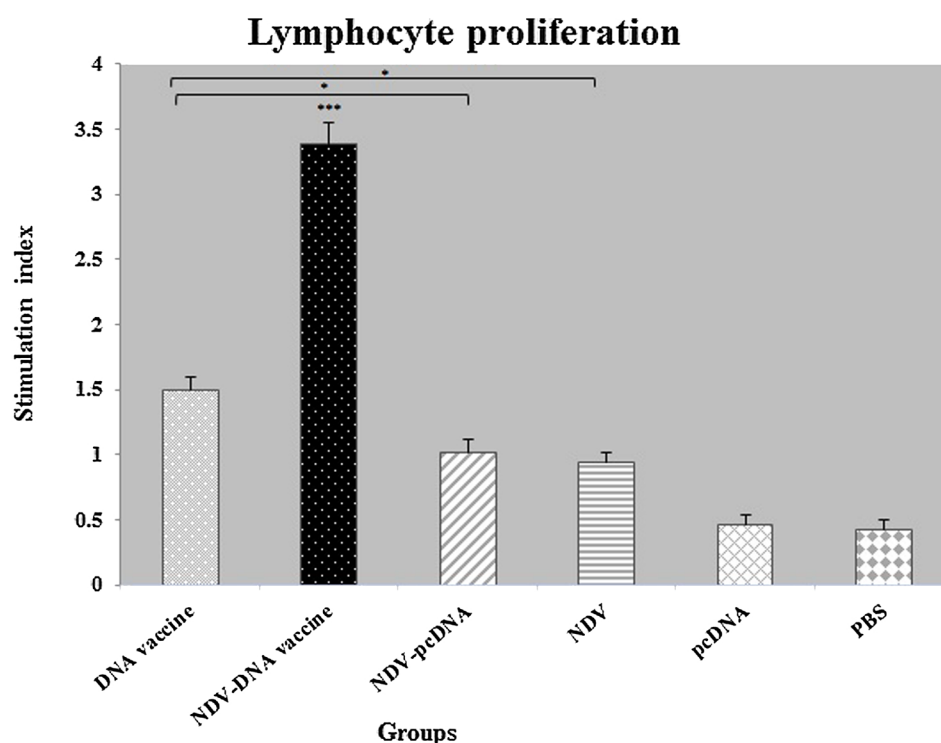
### 3.5. Tumor microenvironment assay

To assess whether the resulting effects of NDV-DNA vaccine on tumor protection could be correlated with the tumor microenvironment factors, the expression of cytotoxicity related proteins (TRAIL and granzyme B) were determined in tumor lysates by ELISA assay (Table 1).

NDV-DNA vaccine group demonstrated a significant increase in granzyme B and TRAIL expression compared to pcDNA3, PBS, NDV-pcDNA and NDV control groups ( $p < 0.001$ ).

DNA vaccinated mice induced significant increase in intratumoral granzyme B and TRAIL expression than control groups, however, the change of TRAIL expression was not statistically significant compared to NDV and NDV-pcDNA3 groups. Furthermore, the results showed that in the tumor lysate that received NDV-DNA vaccine, the expression of granzyme B and TRAIL were 2.07 and 1.9 fold greater than the DNA





**Fig. 2.** Lymphocyte proliferation of experimental groups according to stimulation index (SI). Single splenocyte suspension was isolated one week after the last booster immunization, plated in triplicate in a 96-well plate and stimulated in vitro with HPV-16 E7 epitope. Stimulation index was calculated for all groups. Values are the mean  $\pm$  SD of the mean for the experiments. \*\*\* ( $p < 0.001$ ) indicate statistically significant difference between NDV-DNA vaccine group compared with the other groups as determined by one way ANOVA. \* ( $P < 0.05$ ) shows a statistically significant difference between the indicated groups, as determined by one-way ANOVA. The graph also shows the statistically significant differences between all treatment groups (NDV-DNA vaccine, DNA vaccine, NDV and NDV-pcDNA with PBS and pcDNA3 control groups ( $p < 0.001$ )).

vaccine group ( $p < 0.01$ ). These data suggest that DNA vaccine, HPV-16 E7 gene, that induces intratumoral granzyme B and TRAIL-mediated apoptosis in the context of Tc-1 tumor, can trigger therapeutic T cell and antitumor responses if mice are pretreated with low-dose non-replicating NDV.

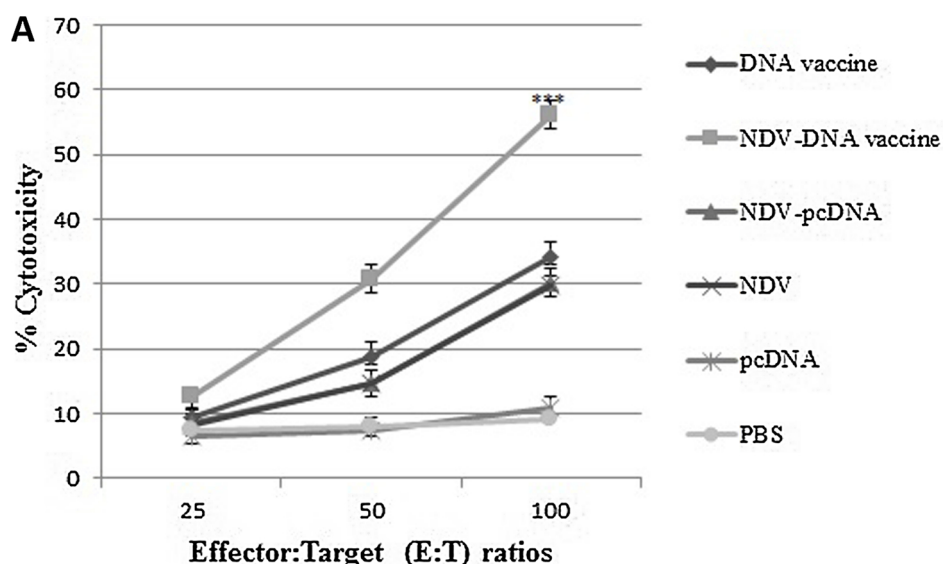
#### 4. Discussion

Virus-induced antitumor responses occur by different mechanisms including direct killing of the tumor cells, induction of tumor cell apoptosis and stimulation of host immune response. Previous study has shown that inactivated and replication-defective NDV demonstrated high rates of lysis in apoptotic tumor cells and great antitumor effects (Yang et al., 2007). The study presented that viral replication does not cause virally induced apoptosis. Furthermore, it has been shown that intratumoral injection of inactivated, replication-defective, Sendai virus particles induced inhibitory effects on the growth of CT26 murine colon carcinoma cells in BALB/c mice (Kurooka and Kaneda, 2007). These data may indicate that antitumor responses mediate in part the increased stimulation of tumor-specific cytotoxic CD8 (+) T cells and inhibition of regulatory T cells, which need to be investigated (Kurooka and Kaneda, 2007). In another study, UV-inactivated, replication-defective Sendai virus particles injected into murine colon carcinoma (CT26) tumors growing in syngeneic BALB/c mice induced systemic antitumor immunity by enhancing NK cell cytotoxicity through secreting IFN- $\beta$  and eradicated 60%–80% of the tumors in renal cell carcinoma tumor-bearing mice (Fujihara et al., 2008). Furthermore, it was demonstrated that low dose of avirulent lentogenic NDV strain, Ulster, cause activation of CTL by augmenting of IFN- $\alpha/\beta$  in metastatic murine lymphoma tumor model (von Hoegen et al., 1990). However, the adjuvant effects of lentogenic NDV on DNA vaccination are not fully understood. Therefore, in the present study, as higher titers of live viruses may cause damages to normal cells (Walter et al., 2012), as well as to evaluate adjuvant effects of lentogenic strain of NDV (Voit et al., 2003), we aimed to analyze immunogenic NDV as an adjuvant for HPV-16 E7 DNA vaccine. Adjuvants can enhance the innate and adaptive immune response to vaccines by interacting with cellular pattern recognition receptors (PRRs) that recognize pathogen-associated

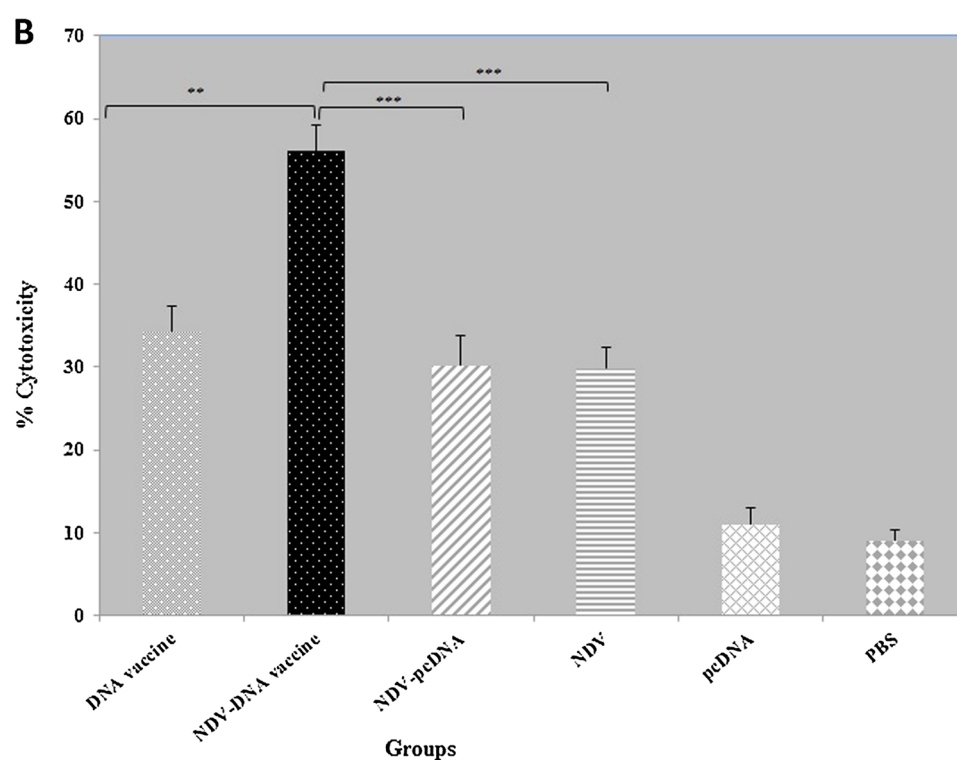
molecular patterns (PAMPs). Several PRRs have been identified in mammals, of which toll-like receptors (TLRs), nucleotide-binding oligomerization domain NOD-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are the most reported (Vasou et al., 2017). Viruses, as previously shown, can be used as immune activator adjuvant for the vaccines (Altinoz et al., 2017; Anzilliero et al., 2014). Activation of PRRs by the immunostimulatory PAMPs (Workenhe and Mossman, 2014), as well as presentation of TAAs to host immune system (Schirmacher, 2015), makes NDV a candidate adjuvant, enabling both arms of immune systems for generation of antitumor responses, or immunological tumor cell death (ICD) (Bastin et al., 2016; Zhao and Liu, 2012).

The description is compatible with previous findings of Kang et al. They showed that inactivated influenza virus can be used as adjuvant for mucosal immunity (Kang et al., 2004). It has been also reported that a lower dose of NDV, as an adjuvant, will lead to completely regression of tumor cells (Lorenz et al., 1994). Therefore, local injection of low dose NDV strain was used to assess tumor-specific immune response.

In the present study, it was demonstrated that antitumor vaccination with a DNA vaccine expressing a tumor-associated antigen E7 from human papillomavirus-16 can be significantly enhanced by treatment with NDV strain. Our results demonstrated that the combination of adjuvanted DNA vaccine with NDV strain conferred strong antitumor responses and suppressed tumor growth in tumor cell (TC-1)-bearing mice. In addition to driving the proliferation of tumor-specific splenocytes, this DNA vaccine adjuvanted with NDV stimulated specific cell mediated immune responses through the induction of high splenic IFN- $\gamma$  and also in inducing E7-specific IL-4 production. In parallel with these results, the splenocyte-derived T cell killing assay also showed cytotoxic activities of cytolytic T lymphocytes against EL4 target cells stimulated with H2b-restricted epitope E7-specific CTL epitope that correlated with enhanced inhibition of the growth of established TC-1 tumor in comparison with treatments using the single agents alone. CTL are generated from precursor T cells following stimulation with cognate peptide presented on target cells in the presence of adequate costimulatory signals and CD4 T-cell help. It has been shown that increased CTL activities are due to CD8 + T cells as a result of cell-cell interaction induced by NDV major immunogenic hemagglutinin-neuraminidase



**Fig. 3.** The combination of NDV-DNA vaccine increases CTL response. Analysis of the cytotoxic activity of CD8 induced by combined group of NDV-DNA vaccine. CTL activity of the lymphocytes from immunized mice (three mice per group) was measured at different E/T ratios by LDH release assay kit (TaKaRa) as described in “Materials and Methods” section. Data are expressed as the mean percentage  $\pm$  standard deviation. Specific lyses of target cells are shown with non-specific background lysis subtracted. \*\*\* ( $p < 0.001$ ) and \*\* ( $p < 0.01$ ) show statistically significant difference between the indicated groups as determined by one way ANOVA. The graph also shows the statistically significant differences between all treatment groups (NDV-DNA vaccine, DNA vaccine, NDV and NDV-pcDNA with PBS and pcDNA3 control groups ( $p < 0.001$ )).



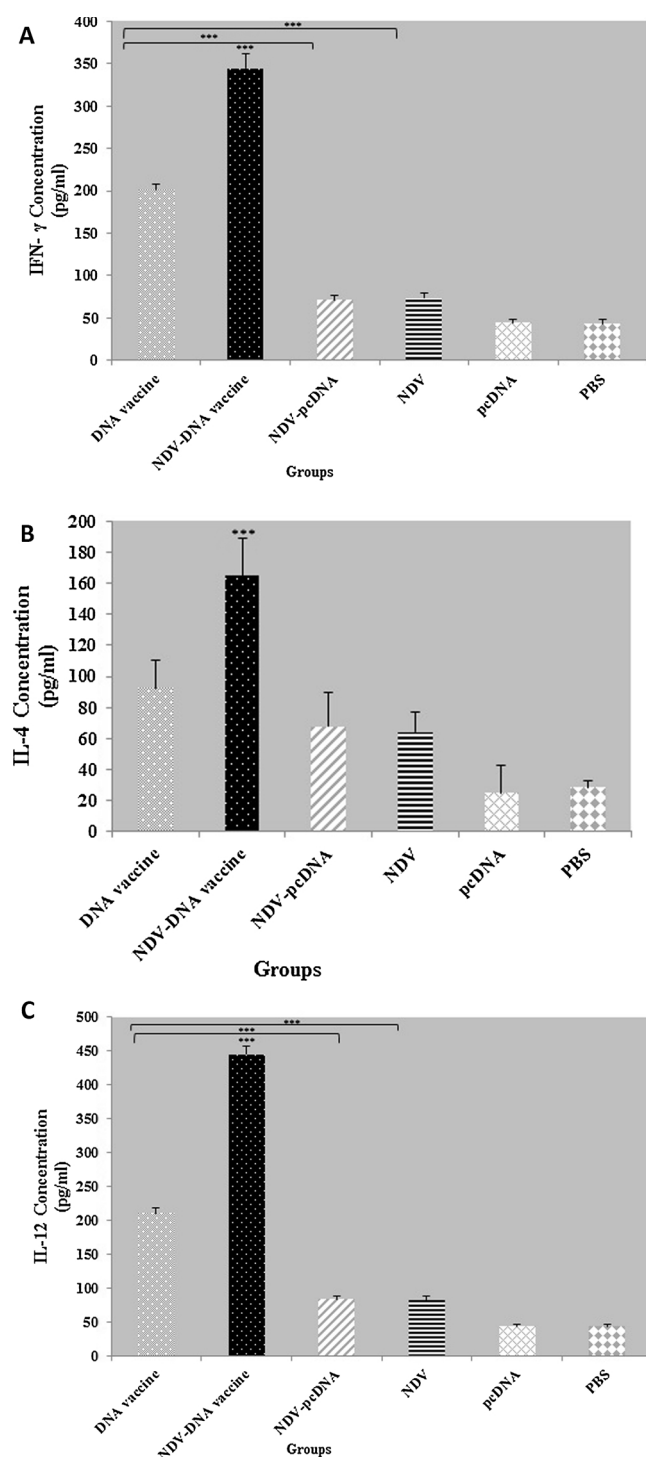
(HN) protein (Sui et al., 2010). Besides, combined E7-DNA -non-replicating NDV (NDV-DNA vaccine group) resulted in enhanced expression of granzyme B and TRAIL in the tumor microenvironment of Tumor-bearing mice.

Adjuvant effects of low-dose non-replicating NDV for triggering innate immune responses may also elucidate the improved efficacy of the DNA vaccine for inducing E7-specific acquired responses in comparison with DNA vaccine alone. Although more experiments with the combined treatment are needed to confirm this hypothesis, our study suggest that PAMP produced by NDV, in combination with DNA vaccine, is likely to enhance IL-12 secretion.

IL-12 is an APC-derived cytokine that stimulates T cells and NK cells to secrete IFN- $\gamma$  and augments the proliferation and CTL activity of these cells. In addition to its important role in the regulation of innate

responses and the Th1 lymphocytes, previous study has suggested that IL-12 is an effective anti-cancer factor against tumors (Tugues et al., 2015; Weiss et al., 2007). Thus, the combined recognition of DNA vaccine and NDV antigens could represent definitive recognition of E7 antigen with an amplified immune response. Meanwhile, our results also showed that the DNA vaccine and NDV groups induced significant cellular immune response compared with control groups.

In parallel with the current results, we have previously demonstrated that vaccines targeting E7 oncogene provide the opportunity to treat HPV-associated malignancies. The studies indicated that DNA vaccine were able to induce specific cellular immunity, which is critical in tumor eradication (Gableh et al., 2016). Cytokine assays showed that activated CTLs could remove tumor cells with E7 antigen (Bahrami et al., 2014; Ghaemi et al., 2012). Besides, the ability of IFN- $\gamma$  and IL-12



**Fig. 4.** IFN- $\gamma$ , IL-4 and IL-12 levels in mice inoculated with NDV-E7 DNA vaccine combination. The cytokines were assayed as mentioned in three different treated mice in those mice previously injected with combined E7-DNA -non-replicating NDV (NDV-DNA vaccine) have significant representing of IFN- $\gamma$  (A), IL-4 (B) and IL-12 (C) secretions. \*\*\* ( $p < 0.001$ ) indicate statistically significant difference between NDV-DNA vaccine group compared with the other groups as determined by one way ANOVA. \*\*\* ( $p < 0.001$ ) also shows a statistically significant difference between the indicated groups. The graph also shows the statistically significant differences between all treatment groups (NDV-DNA vaccine, DNA vaccine, NDV and NDV-pcDNA with PBS and pcDNA3 control groups ( $p < 0.001$ )).

to synergistically augment antitumor immunity (Wigginton et al., 2001) and their role in reducing tumor growth (Gableh et al., 2016) have been confirmed. In addition to inducing effective immune responses, it has been showed that NDV adjuvant of DNA vaccine has increased the induction of TRAIL-mediated tumor cell apoptosis which activates dendritic cells (DCs) maturation to present tumor antigens and cross-prime T-cells. Apoptotic tumor cell death can be immune-stimulatory, as it has been shown that TRAIL-mediated tumor cell killing contributes to immunogenic cell death. The importance of TRAIL was also highlighted by the observation that functional T cells deficiency could be compensated by agonistic anti-TRAIL-receptor antibodies in athymic nude mice (van der Most et al., 2009). Recent evidence suggests that TRAIL is an immune effector molecule and death-inducing cytokine that functions as a selective anti-tumor agent (Valley et al., 2012). TRAIL can be expressed by several immune cells including natural killer (NK) cells, T cells, natural killer T cells (NKT cells), dendritic cells and macrophages (Falschlehner et al., 2009). Significant TRAIL expression was induced on the surface of both CD4(+) and CD8(+) cells upon stimulation with T-cell receptor and type I interferons (IFNs) (Kayagaki et al., 1999). Achard et al reported that attenuated measles virus-stimulated DCs activate TRAIL-mediated cell death and convert DCs into cytotoxic effectors. Acquisition of TRAIL-mediated cytotoxic activity upon exposure to measles virus has been suggested as novel therapeutic strategy for cancer therapy (Achard et al., 2017). In this regards, it has been demonstrated that autocrinely produced IFN- $\gamma$  by CTLs upregulate TRAIL on CTLs, which can kill the tumor cells via TRAIL expression (Ciesek et al., 2008). Therefore, TRAIL play an important role in anti-tumor immune responses (Cretney et al., 2002). These findings support a model in which non proliferating NDV sensitizes tumor cells for TRAIL-mediated killing and antigen specific T cell responses.

The results are supported by previous findings that TRAIL secretion can be induced by nonreplicating viruses. Chaperot et al have shown that after treatment with inactivated influenza virus, plasmacytoid DCs (pDCs) express TRAIL and become able to destroy infected and tumor cells. Induction of TRAIL by the virus is regulated by an autocrine IFN- $\alpha\beta$  loop (Chaperot et al., 2006). The investigation also reported that treatment with influenza virus can turn pDCs into effective cytotoxic cells against TRAIL-sensitive targets (Chaperot et al., 2006). Achard et al also showed that exposure to attenuated measles virus activated TRAIL-mediated cytotoxic functions in DCs and the finding was reported as new potential therapeutic approaches in cancer therapy (Achard et al., 2017). Furthermore, it has been described that tumor-infiltrating pDCs and peritumoral myeloid dendritic cells (mDCs) exert the cytotoxic responses by secreting TRAIL factor and motivate T cell immunity by granzyme B production (Jahrsdorfer et al., 2010; Stary et al., 2007).

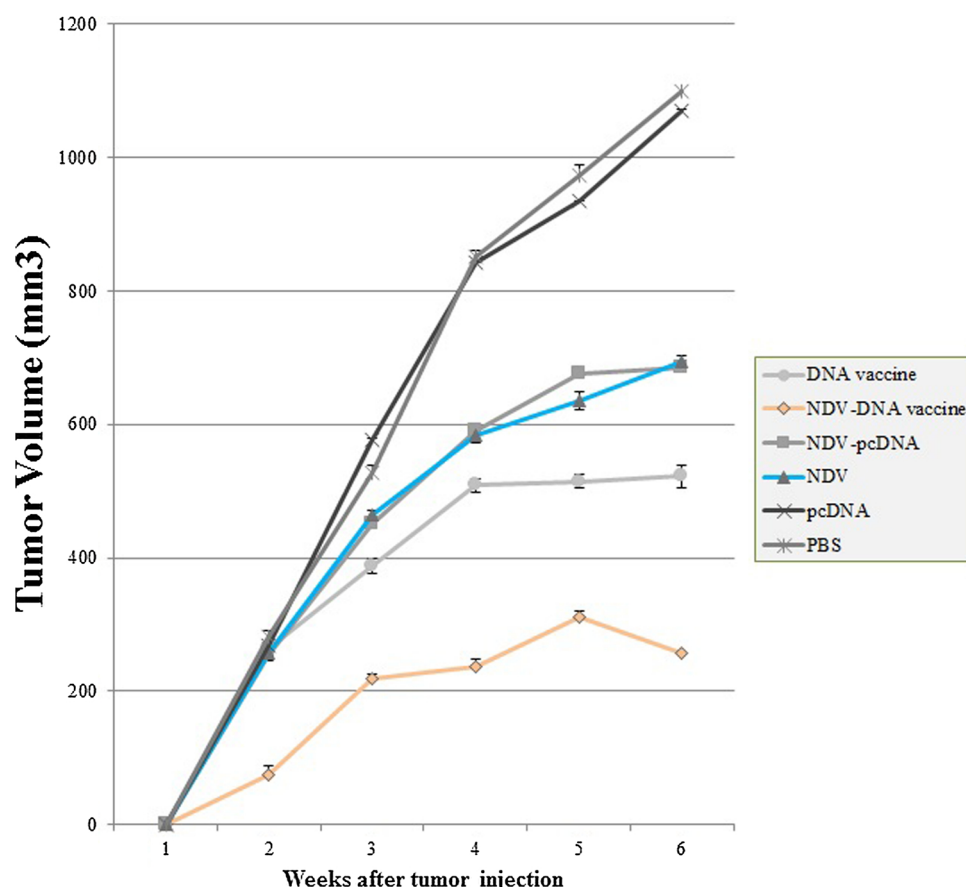
## 5. Conclusions

Given the low immunogenicity of E7 and the role of this oncogene in altering the immune response against infected cells, our results suggest that NDV would be a candidate adjuvant to stimulate both innate and adaptive immune responses alongside E7 DNA vaccine. Our results suggest that fusogenic membrane glycoprotein of NDV may also contribute to enhance a multitude of DNA vaccine-induced antitumor immune response.

Our results showed for the first time showed that NDV adjuvanted DNA vaccine can stimulate tumor-specific immune responses by inducing the cytotoxic mediators and engaging immune effector mechanisms. We believe that the results of our investigation can help to develop efficient therapeutic vaccines based on a synergy between local injection of low dose NDV strain and cancer vaccination.

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**Fig. 5.** In vivo tumor protection experiment in NDV-DNA vaccine mice. An in vivo tumor protection experiment was performed to determine if the observed enhancement in E7-specific CD8 + T-cells led to a significant E7-specific anti-tumor protection response. Tumor growth was monitored by visual inspection and palpation every other days over six weeks. The average tumor size is shown as mean area  $\pm$  SD for each group of mice. Statistical analyses between different groups were performed using Student's *t*-test between different treatments. The data shown here are from one of two representative experiments.

**Table 1**

The levels of cytotoxicity related proteins in a tumor microenvironment. C57Bl/6 mice were challenged with TC-1 tumor cells and vaccinated as described in the text. One week after the last injection, animals were sacrificed and homogenized to form a uniform tumor lysate. The levels of TRAIL and granzyme B in tumor supernatants were analyzed by ELISA. Results are representative of three independent experiments with three mice per group  $\pm$  S.D.

| Groups          | Granzyme B (pg/ml) $\pm$ SD         | TRAIL (pg/ml) $\pm$ SD              |
|-----------------|-------------------------------------|-------------------------------------|
| DNA vaccine     | 206.7 $\pm$ 11.4 <sup>***</sup>     | 159.4 $\pm$ 12.1 <sup>***</sup>     |
| NDV-DNA vaccine | 427.6 $\pm$ 16.2 <sup>***, **</sup> | 318.6 $\pm$ 21.4 <sup>***, **</sup> |
| NDV-pcDNA       | 102.3 $\pm$ 5 <sup>###</sup>        | 125.4 $\pm$ 10.8 <sup>###</sup>     |
| NDV             | 96.2 $\pm$ 9.1 <sup>###</sup>       | 120.8 $\pm$ 11.1 <sup>###</sup>     |
| pcDNA           | 44 $\pm$ 2.9                        | 43.1 $\pm$ 5.4                      |
| PBS             | 41.6 $\pm$ 2.1                      | 38 $\pm$ 5.2                        |

\*\*\* (P < 0.001) indicates statistically significant difference between NDV-DNA vaccine group compared with NDV-pcDNA, NDV, PBS and pcDNA groups.

\*\* (P < 0.01) between NDV-DNA vaccine group compared with DNA vaccine group.

### (P < 0.001) between DNA vaccine group compared with NDV-pcDNA, NDV, PBS and pcDNA groups and also between NDV and NDV-pcDNA groups compared with negative control groups (PBS and pcDNA3) as determined by one way ANOVA.

number: 921) and Research Deputy at Golestan University of Medical Sciences (Gorgan, Iran) Grant number: 35/23551.

### Ethical approval

The animal protocol used in this study was approved by the local animal ethics council of Golestan Ethic Committee of Golestan University of Medical Sciences (ethics number: et- 35/23551). All experimental procedures involving mice were performed in accordance with the national experimental guidelines.

### Competing interests

All authors declare that they have no competing interests.

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